EFFECTS OF 2, 4–DINITROPHENOL ON THE EARLY DEVELOPMENT OF THE TELEOST, ORYZIAS LATIPES

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The stimulating action of 2, 4-dinitrophenol on metabolism and oxygen consumption has been demonstrated for many organisms both embryonic and adult (Shoup and Kimler, 1934, luminous bacteria; Bodine and Boell, 1938, grasshopper embryos; Root and Etkin, 1937, toadfish; and others). It raises the metabolic rate of female rats (Halpern and Hendryson, 1935) but does not accelerate development in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938). In sea urchin eggs this and other nitro- and halophenols simultaneously increase respiration and block cell division (Krahl and Clowes, 1938). The physiological action is discussed by Bodine and Boell (1938).

In this investigation a study has been made of the effects produced by 2, 4-dinitrophenol on the development of the egg of the teleost, *Oryzias latipes,* (*a*) when continued exposure is begun at late cleavage to optic vesicle stages, (*b*) when the chorion is and is not pricked, and (*c*) when the egg is exposed to a lethal concentration for various periods of time.

This substance is highly toxic. In *Rana pipiens* (Dawson, 1938) it either retards, produces abnormalities or completely arrests development depending chiefly on the concentration. Among the abnormalities produced are: persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae. These results are somewhat comparable to what others have obtained in amphibia with high temperature, thyroxine, x-ray exposure, and dilute Ringer's solution. Buchanan (1938) found that such respiratory effects as this substance may have do not compensate for the retarding effect of low temperature on the development of *Amblystoma.* The effects on gastrulation are especially interesting because of the numerous studies of exogastrulation which have been made in both amphibia and echinoderms.

Materials and Methods

Oryzias latipes (*Aplocheilus latipes*) is a small oviparous cyprinodont teleost, measuring about one inch in length, which is also known as the Japanese Medaka. This fish breeds well in the balanced aquarium and can stand low temperatures. Its small, colorless, transparent eggs are laid almost daily over long periods and remain attached to the cloacal region of the female until they are brushed off on the aquarium vegetation. The attachment occurs by the numerous processes peculiar to the egg chorion. Females lay from two or three to as many as twenty-five or more eggs at one time. If the eggs are regularly removed, they will continue to lay for several months. Otherwise the presence of the eggs appears to retard and later to inhibit egg production. Development is rapid and hatching occurs about fourteen to eighteen days after laying. Several factors may be responsible for this variation in hatching time, i.e. temperature possibly, inherent variation in developmental rate of embryos from the same and different females, and the time the eggs are laid in the reproductive period. At the end of the first day after laying, the eggs are in a late cleavage stage. The optic vesicle stage is reached during the second day and on the third to fourth days the heart is beating and differentiation is well advanced.

For the present study a 1 per cent stock solution of 2, 4-dinitrophenol was made according to the method of Halpern and Hendryson (1935), and sufficient amounts added to spring water to make the desired solutions. Concentrations of 1 : 10,000 up to 1 : 1,000,000 were used, but the most significant results appeared at concentrations from 1 : 40,000 up to 1 : 200,000. For the most part the embryos remained in the solutions for the entire experimental period. Eggs were also placed in a known lethal concentration for the late cleavage stage (1 : 10,000) and samples removed at intervals thereafter to fresh water. Since the egg is surrounded by a chorion, several studies were made in which the chorion was pricked. All experiments were made at room temperatures. Exposure was begun at four developmental stages; early and late cleavage, closure of the blastopore, and optic vesicle.

Experimental

In general the most conspicuous effect upon development is a retardation in both growth and differentiation which is proportional to the length of exposure and to the concentration of the drug, but there is variation in different individuals of a culture as some are more susceptible than others. No evidence of any stimulation was seen either in the developmental rate or in the hatching time nor were any cases

of exogastrulation obtained. The results obtained in one experiment are shown in Table I which is a typical example of the many that were

TABLE I

Effect of 2, 4-dinitrophenol on early development of *Oryzias latipes* (Late cleavage stage)

Experiment	Concentration	Age in days	Number dead	Average heart-beat (No. of secs. for 50 beats)	Heart-beat (low and high)	Temperature when examined	Comments	Hatching time
21	Control	7	None	28.7	24 34	25° C.		1 in 18 days 1 in 21 days 5 in 25 days
	1 : 40,000	7	None in 8	35.2	33 42	25° C.	Markedly retarded. Few have no circulation and rudimentary heart.	All dead 13 days after laying.
	1 : 80,000	7	1 in 14	31.2	27.5 34.5	25° C.	3 retarded, others like control.	2 dead in 22 days, all dead in 25 days, none hatched.
	1 : 120,000	7	None in 15	29	26 32	25° C.	6 retarded	2 in 19 days 2 in 21 days 1 in 22 days 2 in 25 days All died soon after hatching.
	1 : 160,000	7	None in 14	28.6	26.5 31	25° C.	No retardation	1 in 18 days 8 in 19 days 2 in 21 days 1 in 25 days 2 died
	1 : 200,000	7	1 in 7	28.4	27 30	25° C.	No retardation	1 in 18 days 5 in 25 days

tried. Concentrations greater than 1 : 10,000 inhibit all further development beyond possibly a few cleavages, while in a solution of 1 : 160,000 development is normal and the embryos hatch about the same time as the control. Although development occurs in concentrations greater than 1 : 90,000, it is very retarded, causing early deaths and no hatching. The effect of the drug is cumulative with time and retardation becomes increasingly evident with continued exposure to strong concentrations. These observations confirm those of others on the high degree of toxicity of this substance.

Except for the difference in the effective concentrations and the formation of certain abnormalities, the effects of 2, 4-dinitrophenol on the development of this fish closely duplicate those found by Dawson on the frog, and by Solberg (1938) for *Fundulus* embryos exposed to X-rays. The survival of the embryos is directly correlated with their

sensitivity to the drug.   It is generally difficult to duplicate the results of one experiment with eggs taken from the same or different females on successive days.   Another comparable observation is that temporary developmental inhibition does not necessarily cause irreversible injury, providing the exposure is not too long.

Both the controls and the experimental animals were run at the same temperature.   This was always that of the laboratory and it varied between 24° and 27° C., with an average around 25°.   This slight variation probably did not influence the action of the drug since Dawson found that in the frog the results were apparently not greatly modified by temperature.   However, a large proportion of his animals showed arrested and abnormal development at 6° to 12° C.   This is far below the temperature range of the present study.   In *Bufo,* Buchanan found that the toxicity is higher at 21° than at 6° C.

The heart and extra embryonic vascular system are particularly sensitive.   Since the rate of heart beat corresponds roughly to the concentration and length of exposure, it serves as a check on the other effects observed.   However, no stimulation was observed.   Concentrations greater than 1 : 130,000 progressively slow up the rate and amplitude of the heart beat and also provoke structural irregularities.   Deformities of the heart lead to interrupted and poor circulation which in turn provokes other abnormalities as development progresses.   These embryos die early.   In concentrations greater than 1 : 40,000 the extra-embryonic circulation never becomes established and the heart is rudimentary although other embryonic differentiations may take place, i.e. nervous system, sense organs, body form, and sometimes somites. Blood corpuscles develop in all embryos but the quantity is less in the more retarded types.   Blood vessels are also affected as, in the extra-embryonic area especially, some may be distended with blood while others remain unconnected.

Deformities in the myotomes and a reduction of their number are of common occurrence.   The number may especially be reduced in the tail.   Here the irregularities bring about shortening and curious shapes and positions of the tail.   It often becomes bent at odd angles, twisted, curved or shortened.   The tail fins may fail to develop but typical paired appendages are usually present in less abnormal cases.   Embryos of these types did not hatch.

The nervous system and sense organs exhibit a variable response to the drug.   In the stronger solutions some development stops at a neural tube stage while other embryos show the presence of the optic vesicle with but slight indications of the brain.   In others the nerve cord is shortened or deformed.   Often the neural canal is filled with a mass

of cells. Different regions of the brain are often missing, enlarged or abnormal on one side or the other. Very little effect is observed on the ears. The optic vesicles often fail to invaginate or show a variable amount of pigmentation. A size difference is also noticed as well as a malformation of the lens.

No particular type of teratological embryo is produced by dinitrophenol, but the production of uniform abnormalities is fairly consistent. Gastrulation either occurs normally, is retarded, or is entirely inhibited. If the exposure is not too long a few of the retarded embryos

TABLE II

Sensitivity of different developmental stages to a lethal solution of
2, 4-dinitrophenol (1 : 10,000)

Stage	Length of exposure before transfer to fresh water					Development during exposure
	4 hrs.	10 hrs.	21 hrs.	29 hrs.	45 hrs.	
Early cleavage	Retarded in size and development.	Small optic vesicle or rudimentary nerve tube. Some dead.	Dead			None
Late cleavage	Retarded in size and development.	Small optic vesicle stage.	Small optic vesicle stage.	Dead		None
Closure of blastopore	Slight retardation in some.	Smaller and thinner embryos. Slight pigmentation of eyes.	No development beyond optic vesicle or young non-pigmented optic cup stage. Rudimentary heart. No circulation.	No development beyond young optic vesicle stage.	Dead	None. Perhaps slight increase in cell number.
Optic vesicle	No effect		Slight retardation. Slower heart rate.		Marked retardation. No heart action. 96 hrs. exposure did not inhibit development.	Yes, but at a slower rate.

The above results are those seen after the embryos had been in fresh water for 3–4 days following exposure.

are able to complete gastrulation and to continue development in fresh water. Otherwise they give cases of persistent yolk plug which also occur in some instances during the experiment. Subsequent development is very atypical and death soon ensues. The inhibited blastulae do not increase in size and development goes no farther except for a few irregular cleavages especially noticeable at the periphery. None of these are able to gastrulate in fresh water. Unlike the frog, no living cases of exogastrulation were seen.

The results of the study of the relative sensitivity of several developmental stages to dinitrophenol are summarized in Table II. They show that the sensitivity decreases at least from the early cleavage stage to the optic vesicle stage and in the latter case development is able to continue, but more slowly, in a lethal solution which quickly kills the earliest stage tested.

DISCUSSION

The resistance of fish embryos to the experimental conditions described in this study varies at different stages. At the optic vesicle stage they are much less sensitive to 2, 4-dinitrophenol than at the early cleavage stage. Solberg (1938) has likewise observed this to be true of *Fundulus* embryos exposed to X-rays. The earlier in development that the embryos are treated, the greater are the deformities. During the gastrula stages there is again a slight increase but thereafter the sensitivity declines rapidly and larger doses are required to modify development. Changes also occur in the sensitivity of different organs of the fish embryo to X-rays and dinitrophenol. As differentiation progresses the organs become more resistant; the time depends on the time of appearance and the rate of differentiation.

There was no special difference noted in the adjustment of early and late cleavage, or even older stages to the experimental solutions. Except for the question of sensitivity, early and late stages gave almost comparable effects. Dawson (1938) found this the case in his study of the effects of dinitrophenol on the development of *Rana pipiens* when treatment was begun at the 2-cell and early blastula stages. However, under the influence of high temperature, Hoadley (1938) found that the behavior of frog eggs is different immediately after the first cleavage from what it is at the 128-cell stage. There was a more complete adjustment to the new conditions on the part of the first group.

The suppression of cell division by dinitrophenol appears to be a common phenomenon. First reported (Krahl and Clowes, 1938) for sea urchin eggs, it has subsequently been found to occur in the frog (Dawson, 1938) and in the present study on the fish. At first this effect is hardly noticeable but with continued exposure mitosis is almost suppressed. The rate of this disturbance depends on the concentration of the drug. At one extreme development may be stopped at such an early stage as the optic vesicle, while at the other quite typical embryos may develop. However, the latter are noticeably smaller than the control, die sooner, and never hatch. Solberg (1938) finds that following X-ray exposure, the mitotic index of *Fundulus* embryos changes which accounts, at least in part, for the changes in sensitivity. The close

parallelism of the two changes during early development seems to him to be more than coincidental.

The chorionic membrane of the *Oryzias* egg is permeable to dinitrophenol but some difference is noted in the effective concentrations between cases of whole membranes and those in which the chorion is pricked. Even further differences might be noted if this membrane were entirely removed. The drug evidently penetrates slowly and higher concentrations and longer exposures are necessary for the intact membrane. This may account for the difference in the effective concentrations reported for the frog and *Bufo*.

In vertebrates, studies of exogastrulation have been limited thus far to the amphibia. The period of gastrulation is a critical one. Hoadley (1938) has shown that under the influence of supra-maximum temperatures early gastrulae and very late blastulae are unable to complete gastrulation and die. Early blastulae form giant blastulae which never undergo gastrulation. Many other environmental conditions are known to affect or modify amphibian gastrulation (cf. Dawson, 1938); dilute Ringers solution, X-ray exposure, thyroxin. Dawson obtained persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae through the use of 2, 4-dinitrophenol. These results show that exogastrulation in amphibia is no more of a specific reaction to any particular agent than has been found to be the case among echinoderms (Child, 1936). They have all followed instances of developmental disturbance and arrest.

In the present study no cases of living exogastrulae were seen. Either gastrulation was inhibited entirely and the persistent blastulae remained as such until cytolysis and death occurred or gastrulation was retarded or stopped during the process. Cases of persistent yolk plug occurred occasionally. In all these interrupted types, the origin and the differentiation of the organ rudiments and even body formation were restricted. There was no indication of a differential effect on gastrulation alone. It would have been interesting to see what form exogastrulation would take in a telolecithal egg.

Certain types of abnormalities occurring in the embryos under the influence of 2, 4-dinitrophenol have been reproduced in fish by other agents and in the amphibia by various agents. For example, in *Fundulus heteroclitus* Solberg (1938) obtained comparable effects with X-rays on the development of the circulatory system, myotomes, nervous system, etc. Reference should be made to Dawson (1938) for work on the frog embryo. In general no special type of teratological embryo was produced but results were fairly consistent. As Dawson says, none of the effects obtained by treatment with dinitrophenol can be inter-

preted as being produced specifically by the drug but are such as might follow any treatment which disturbs the orderly processes of development and differentiation. If the increase in metabolic rate is one of the factors involved it is difficult to correlate this idea with the results secured by X-rays and by other agents tested on amphibia.

From the present study it would appear that 2, 4-dinitrophenol does not stimulate gastrulation or development in *Oryzias latipes* and, in the effective concentrations employed, it acts like any toxic agent. As such it retards, inhibits and disturbs normal orderly developmental processes in proportion to the concentration and length of exposure. Its effects are cumulative with time. Various concentrations which did not produce any observable defects caused no reduction in the hatching time. This confirms in the fish the observations of others that the drug does not accelerate development or metamorphosis in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938).

Dawson suggests that the increase in the metabolic rate may be one of the factors involved in the effects produced by 2, 4-dinitrophenol on the frog embryo since they resemble those caused by thyroxin (Baumann) and high temperature. According to Bodine and Boell (1938) the toxic effect may be due to the increased accumulation of toxic metabolic products of which the organism is unable to rid itself. In this connection it is interesting to note the conclusion of Lindahl (1936) from his study of the effect of SO_4-deficient sea water on the development of *Paracentrotus lividus* (the European sea urchin), since results were secured which in some cases are comparable to those of the frog and fish. SO_4-deficient sea water produces persistent blastulae, exogastrulae, ectoblastulae with cilia, etc. He considers that hydrocarbon metabolism dominates at the animal pole while protein metabolism dominates at the vegetal pole. The toxic action of this deficiency is caused by the accumulation of toxic phenolic derivatives coming from protein metabolism.

Summary

A study has been made of the effect of 2, 4-dinitrophenol on the development of four embryonic stages of the teleost, *Oryzias latipes,* i.e. early and late cleavage, closure of the blastopore and optic vesicle stages. Both the sensitivity of the whole organism and constituent parts to the drug decreases with age and differentiation. The general effect is cumulative and proportional to the concentration and length of exposure. In addition to inhibitory and retardative effects, other developmental abnormalities include those of the heart and blood vessels, myotomes, nervous system, body shape, etc.

Concentrations of 1 : 10,000 up to 1 : 1,900,000 were used, but the most significant results appeared at concentrations from 1 : 40,000 to 1 : 200,000. When the chorion is pricked there is some difference in the effective concentrations. No evidence of any stimulation was seen either in the developmental rate or in the hatching time. Gastrulation was either inhibited or retarded and no examples of exogastrulation were seen. If exposure has not been too long, recovery takes place to a more or less extent in fresh water.

Some of the results of the present study have been duplicated in the amphibia by various agents and in fish by X-rays. In general no special type of teratological embryo was produced but the results are fairly consistent and reproducible. It does not seem possible to attribute the effects to a specific action of this drug and hence to increased metabolic and respiratory rates alone, unless the change produces toxic products of which the embryo is unable to rid itself.

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